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FIBER-OPTIC SPECTROPHOTOMETRY OF COWPEA NODULES TREATED WITH NITRATE OR AMMONIUM

KEY WORDS: Ammonium, Cowpea (Vigna unguiculata [L.] Walp.), Fiber-optic spectrophotometry, Nitrate, Nitrogen fixation inhibition, Rhizobium, Root nodule.

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ABSTRACT: Cowpea nodules (Vigna unguiculata [L.] Walp. cv. CB5) were pierced with two opposing optical fibers (280 μm diameter), and absorbance spectra of 0.1 mm of nodule tissue were recorded from 415 to 600 nm using a modified spectrophotometer with a nodule sampling stage.

The nodule spectra exhibited two absorbance bands, a major band in the near-UV (415-450 nm) and a lesser one in the green-yellow region (510-585 nm); the latter exhibited a prominent peak at 550 nm. Nodule spectra were consistent with the superposition of the spectra of ferroleghemoglobin (Lb^{2+}), oxyleghemoglobin (Lb^{2+}- O_2), and cytochrome c (550 nm). The detection of leghemoglobin in vivo was confirmed by demonstrating the reversibility of binding of CO to Lb, and by comparing the spectra of live nodules with those obtained from anaerobic leghemoglobin preparations.

The effects on the nodule spectra of two successive applications (36 and 39 days after planting) of 5 and 10 mM NO_3^- or NH_4^+ to the nutrient solution

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bathing the nodulated roots were determined. The spectra of NO_3^-- and NH_4^+-treated nodules were indistinguishable; in each case the height of both the near-UV and green-yellow absorbance bands decreased with treatment. Treatment with N caused a significant reduction in the area of the green-yellow absorbance band.

The spectra of green leghemoglobin pigments were obtained from senescent nodules; these exhibited a reduction in the height of the near-UV absorbance band and a flattening of the green-yellow band similar to that of nodules treated with N, but the broadening of the near-UV band was greater in green nodules.

INTRODUCTION

Leghemoglobin forms a stable, reversible complex with oxygen (1, 2); it facilitates oxygen diffusion to bacteroids, and acts as a buffer to maintain the correct oxygen tension at the bacteroid membrane (3, 4). Nodule leghemoglobin content has been correlated with nitrogen-fixing ability (5, 6, 7, 8), and with nitrogenase activity as measured by acetylene reduction (9, 10, 11).

The usual field method of assessing nitrogen fixation by examining root nodules for the presence of leghemoglobin is as sensitive an indicator of nitrogen fixation as presently exists (12). Quantitative spectroscopic observation of nodule central tissue is difficult (2), and development of methods is proceeding along different routes (13, 14). Early spectroscopic studies using a chamber constructed from microscope slides to hold nodule slices equilibrated with gas mixtures of various oxygen tensions demonstrated the progressive oxygenation of nodule leghemoglobin as oxygen tension was increased (2). A chamber with a removable side plate was used to equilibrate nodule segments with Ar, N_2, and CO and show that visible-light absorbance spectra of legume root-nodule central tissue under near-physiological conditions represent the superposition of the spectra of leghemoglobin and cytochrome c (15). The diffuse absorbance band between 530 and 570 nm was attributed to uncomplexed ferroleghemoglobin (Lb^{2+}) and the sharp peak at 550 nm to cytochrome c (15). Cytochrome c is a component of the bacteroid respiratory electron transport system (16, 17). Thus nodule spectra can provide a window into the respiratory apparatus of nodules.

More recently, disruption of nodule central tissue has been avoided by examining entire nodules and nodule segments in situ by carefully positioning

them adjacent to the photomultiplier. This technique works best on flat, elongated nodules; its use is limited to small, well positioned nodules from a restricted range of legume-Rhizobium associations (13).

The inhibitory effect of high inorganic nitrogen concentrations in the rooting medium on the growth and nitrogen-fixing activity of root nodules has been the subject of much study, but the mechanism of inhibition is still not clear (18, 19,20, 21, 22, 23, 24, 25, 26). Because nitrate is the predominant form of nitrogen available to plant roots growing in soil, its inhibitory effect has received more attention than that of ammonium. The specific inhibitory effect of nitrate on nitrogen fixation has been postulated to occur because "nitrate forms a NO-compound with leghemoglobin" (27). Nitrite strongly inhibits the nitrogenase activity of soybean bacteroids (28), and causes a rapid deoxygenation of leghemoglobin followed by autooxidation and consequent inactivation of the hemoprotein (29). However, an NADH-dependent leghemoglobin reductase active in soybean nodules restores ferrileghemoglobin (Lb^{3+}) to the active ferrous form (30); this may explain the absence of oxidized leghemoglobin in vivo.

By inhibiting nitrogen fixation in nodules with nitrate or ammonium, and observing in nodule tissue spectral changes caused by treatment with inorganic nitrogen, it should be possible to determine if the mechanism of inhibition of nitrogen fixation by nitrate involves the rapid inactivation of leghemoglobin. In contrast, any effect due to the inhibition of synthesis of macromolecules will have a much longer time lag, on the order of several days (18). One objective of the present study was to distinguish whether the repression of nitrogenase activity by nitrate is due to the rapid inactivation of leghemoglobin by nitrite, or results from the much slower inhibition of nitrogenase and leghemoglobin syntheses.

MATERIALS AND METHODS

Plant Culture

Cowpea seeds (Vigna unguiculata [L.] Walp. cv. CB5) were surface-sterilized by immersion in 95% ethanol for 30 s and then in 3% calcium hypochlorite solution for 10 minutes. The seeds were rinsed six times (2 min per rinse) with sterile water. The seeds were sown in a metal tray containing moist

sterile vermiculite, and were inoculated by lightly dusting them with peat-based inoculum (cowpea Rhizobium strain 176A32) prior to planting. The tray was covered with polyethylene film and incubated in the dark for 48 h. After incubation, the tray was placed under artificial light (see below) until the seedlings emerged and reached the two true-leaf stage. Twelve seedlings were selected for uniform size and transferred into 10 L pots which were placed on the laboratory bench top.

The pots contained aerated nutrient solution composed of the following: 1 mM KNO_3, 2 mM $MgSO_4$, 0.2 mM KH_2PO_4, 4 mM K_2SO_4, 2 mM $CaSO_4$, 51.8 µM B, 9.1 µM Mn, 0.76 µM Zn, 0.31 µM Cu, 0.10 µM Mo, and 35.8 µM Fe (EDDHA). The nutrient solution was changed weekly and the pH was maintained at approximately 7.0. Care was taken to keep the solution in contact with the crown root nodules.

The plants were illuminated with model M400-U metal halide lamps (Sylvania Inc., Manchester NH) (85% of total intensity) and "Vitalite" fluorescent lamps (Duro-Test Co. Daly City, CA) (15% of total intensity). The photon flux density was approximately 300 µmol / m^2 s at pot level. The daylength was 16 h, and day and night temperatures measured at mid-canopy were 30 °C and 21 °C, respectively. Nodules were large enough for spectral analysis at 36 days after planting (DAP). One nodule from the crown area of each plant was pinched off, and its spectrum was recorded as described. Care was taken to sample healthy nodules from the entire circumference of the crown.

After the plants were sampled, the pots were assigned treatments in a completely randomized design. Treatments consisted of the addition of sufficient calcium nitrate or ammonium chloride to yield a 5 mM concentration in the nutrient solution. A second pulse of 10 mM nitrogen in the same form was added at 39 DAP. Two plants that were poorly nodulated were used instead for the CO equilibration experiment (see below). A total of four reference and six nitrogen-treated plants were sampled until 55 DAP.

Fiber-optic Spectrophotometry

A model EU 700 GCA-McPherson double-beam digital spectrophotometer was modified by substituting two one-meter by 0.28 mm diameter plastic optical fibers (Edmund Scientific Co., Barrington, NJ) for the normal light paths (Fig.

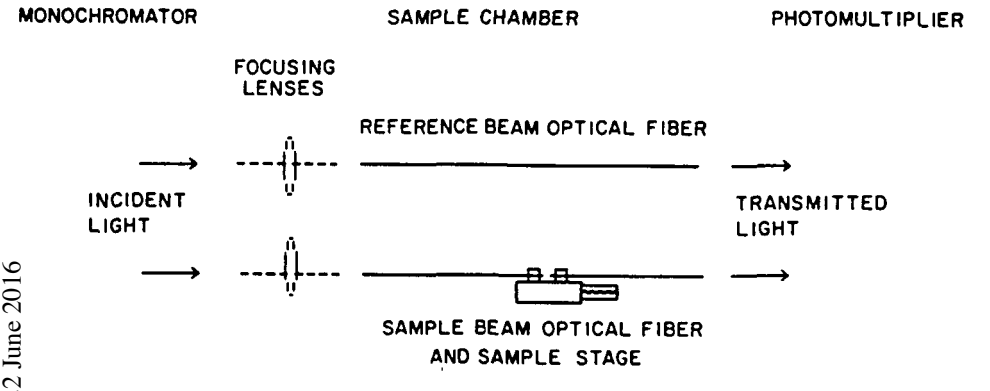

FIGURE 1. Schematic Diagram of the Spectrophotometer Optical System Modified for Operation with Optical Fibers.

1). The monochomator reference and sample slit beams were focused with convex lenses at the input ends of the light guides. The sample and reference light guide output ends were directed at the photomultiplier tube. An optical mini-translation stage (Daedal Inc., Harrison City, PA) with micrometer drive was adapted for sampling nodules by rigidly mounting two syringe needle-holders on it (Fig. 2). The syringe needle ends were cut flat and smoothed with a knife file. The sample beam light guide was cut at the midpoint, and the tips were polished and flared with the heat from a soldering iron so that they could be held in 20 gauge syringe needles. The application of heat caused the optical fiber tip to enlarge and become hemispherical, thus preventing the fiber from moving back into the syringe needle. (Silica optical fibers can be ground flat, thus reducing light scattering from the fiber end. However, silica optical fibers are brittle and protected by a plastic sheath, and we were unable to secure them within the syringe needles to give a reproducible light path because of slippage between the optical fiber and its sheath.) The two needle-holders were machined from a single piece of aluminum and secured on the translational stage with screws so that the needle ends remained parallel and opposite. Light transmission through the sample beam light path was maximized by aligning the optical fiber ends to obtain the highest possible light intensity at the photomultiplier.

The micrometer reading when the optical fiber ends were just touching was obtained under a microscope. The optical fibers were then driven into the nodule

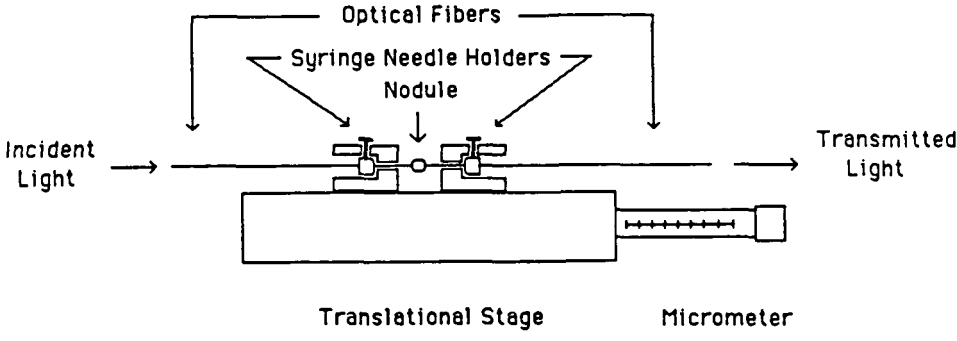

FIGURE 2. Schematic Diagram of the Stage Used for in vivo Examination of Root Nodules.

central tissue by advancing the micrometer drive to give a light path of ~ 0.1 mm. The nodule central tissue was protected from atmospheric oxygen by coating the surface of the needles with silicone vacuum grease to seal the puncture.

Root nodules were scanned from 415 to 650 nm; the slit width was 1 mm, and the spectrophotometer was set to scan in 1 nm increments and average the signal from each increment for 400 ms. Approximately 3 min elapsed from the time of initial needle insertion to the end of the scan. The absorbance baseline was not completely flat, and the spectra were corrected by manually digitizing the curves and subtracting the baseline. The area of the 510-585 nm absorbance band was measured by tracing the curve on a digitizing tablet (Apple Computer, Cupertino, CA). The digitizing tablet drew a straight line between the end points (at 510 and 585 nm) and integrated the area under the curve.

Acetylene Reduction Assay

Nodules were assayed for acetylene reduction activity immediately after the spectra were obtained. Each nodule was placed in a 5 mL syringe into which 1 mL of acetylene and 4 mL of air were drawn. The nodules were incubated in this 20% acetylene atmosphere for 30 min. The gas sample was then injected into a 10 mL evacuated container. The syringe was rinsed with 5 mL of air and the rinse was also injected into the container. After the acetylene reduction assay, the nodules were dried in a forced-draft oven at 60° C before weighing. Ethylene was

determined on a Varian 3700 gas chromatograph equipped with a flame ionization detector (340 °C), using a Porapak T column (2 m X 1.6 mm i. d.) at 80 °C with N_2 carrier gas at a flow rate of 25 mL/min.

CO Equilibration

The reversibility of CO binding to leghemoglobin was demonstrated by incubating excised root systems of two surplus 36-day-old plants for 10 min alternately in air, CO, O_2, and CO in a polyethylene bag under a safety hood. Because of safety considerations, no attempt was made to construct a gassing chamber for equilibration of intact nodules. The spectra of several nodules picked at random were recorded after each equilibration.

Anaerobic Nodule Cytosol Preparations

Ten grams of nodules were suspended in 15 mL each of 0.2 M ascorbate and 0.1 M phosphate buffers (pH 7.0) plus 3 g of polyvinylpyrrolidone, and crushed under Ar. The homogenate was centrifuged at 500g for 5 min to remove cell debris, and the pellet was discarded. The supernatant was centrifuged at 10,000g for 10 min to sediment bacteroids and mitochondria, and the pellet was again discarded. A micro-sample chamber was constructed for the spectrophotometer by drilling a T-shaped compartment (300 μm diameter) into a piece of Lucite stock (5 mm square). To use the micro-sample chamber, the optical fibers were driven into the compartment along the arms of the T to give a 1 mm light path, and the nodule supernatant was injected through the base of the T. Anaerobic conditions were maintained during spectral determinations by placing the sample stage with micro-sample chamber in a polyethylene bag sparged with N_2.

RESULTS AND DISCUSSION

Analysis of Nodule Spectra in Air or O_2 Atmosphere

The air- and O_2-equilibrated nodule spectra were essentially identical, and exhibited absorption bands in the near-UV and green-yellow region of the spectrum. The visible absorption band extended from 510 to 585 nm and exhibited a small, relatively sharp peak at 550 nm (Fig. 3). The visible light spectra are similar to those obtained from N_2- or air-equilibrated nodule segments

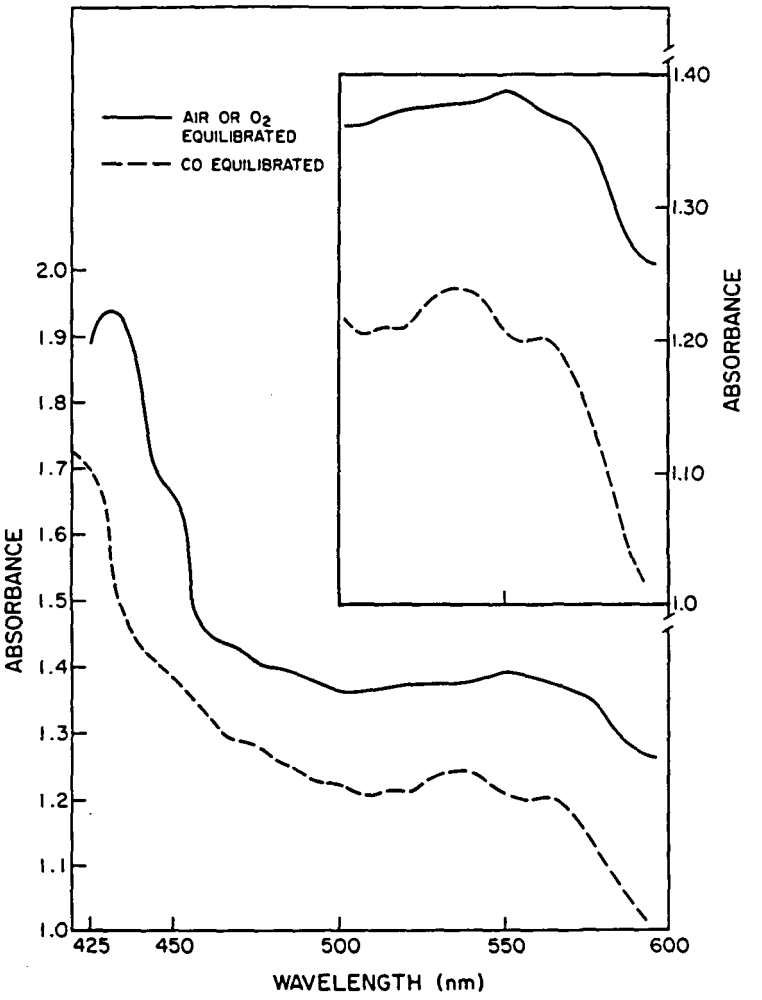

FIGURE 3. Absorbance Spectra of Nodules Demonstrating the Reversibility of CO binding. The Solid Trace Represents the (Identical) Spectra Obtained Both Prior to and After Equilibration with CO. Excised Root Systems were Equilibrated for 10 min. Alternately in Air, CO, O_2, and CO in a Polyethylene Bag.

(15), and appear to be a superposition of uncomplexed Lb^{2+} (a broad absorbance band from 530 to 570 nm), Lb^{2+}-O_2 (peaks at 542 and 572 nm), and Rhizobium cytochrome c (peak at 550 nm). The similarity between our O_2- and air-equilibrated spectra indicate that our 10 min equilibration was insufficient for complete oxygenation of Lb, since others have recorded different spectra for O_2- and air-equilibrated nodules following 30 min equilibration with O_2 (13).

Ferrileghemoglobin, which has a weak absorption band in the 630 nm region, was not detected in nodule spectra, and attempts to observe this band by in vivo oxidation of leghemoglobin resulting from injections of 100 μM $K_4Fe(CN)_6$ into nodules were unsuccessful. The Lb^{3+} absorbance band may have been obscured by wavelength-dependent light scattering.

The absorbance of nodule tissue is much greater at 415 nm than at 600 nm in part because of greater light scattering at the shorter wavelengths. Light scattering in nodule tissue has been compensated by passing the reference beam through milk (15). The fiber-optic spectrophotometer permitted the use of a short light path (0.1 mm) which minimized light scattering, and the significant features were apparent in the raw spectra. The visible absorbance band appeared rounded and diffuse; however, a milk reference apparently produced artifacts and the results were non-reproducible (unpublished data). An alternative technique (which we did not perform) is to record the spectra and deduct the component due to light scattering as estimated from calculations. The spectrum of crude nodule supernatant recorded under anaerobic conditions (Fig. 4) exhibited a broad absorbance band from 510 to 610 nm with shoulders at 540 and 570 nm. This spectrum is similar to that of intact nodules (except for having much less of a contribution from light scattering), and appears to be a superposition of the spectra of Lb^{2+} and Lb^{2+}-O_2. The 550 nm peak of cytochrome c is absent as would be predicted since the bacteroids were removed by centrifugation.

Effects of CO

When air- or O_2-equilibrated nodules were equilibrated with CO, a prominent absorption peak appeared at 536 nm with a shoulder at 562 nm, indicating the formation of Lb-CO (Fig. 3). Although there was more variability in the spectra of later equilibrations, replicate spectra from each equilibration were similar. The reversibility of the spectral changes is indicative of the contribution of leghemoglobin to the spectra (2).

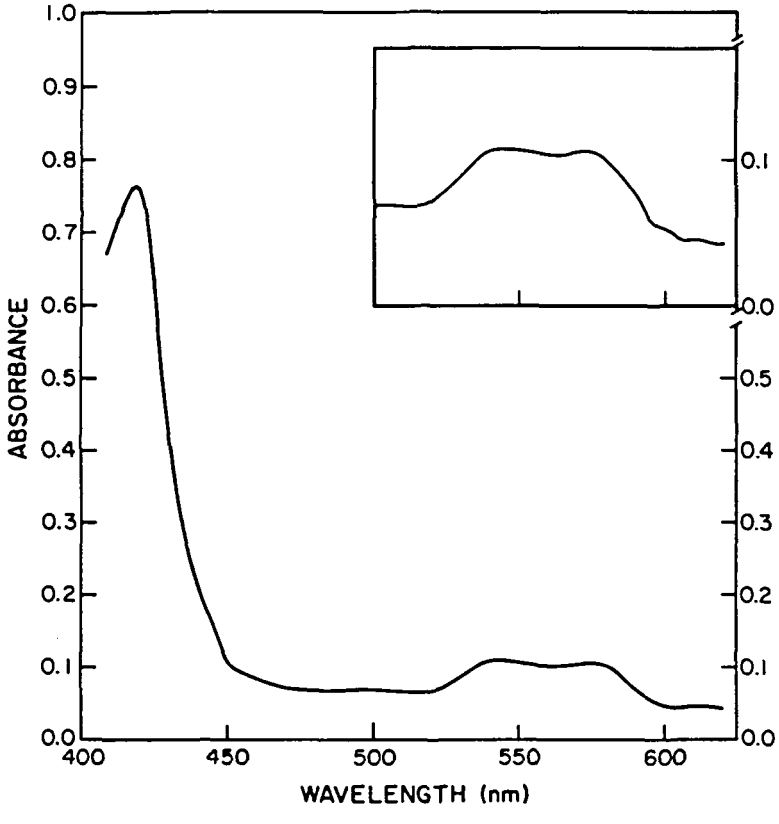

FIGURE 4. Absorbance Spectrum (1 mm Light Path) of Crude Supernatant from Nodules Crushed in 0.2 M Ascorbate and 0.1 M Phosphate (pH 7.0) Prepared and Centrifuged Under Ar. The Spectrum was Recorded Under Anaerobic Conditions Using the Sample Stage With Micro-sample Chamber Placed in a Polyethylene Bag Sparged with N_2.

Effects of Inorganic N

Changes in nodule spectra due to two pulses (5 mM and 10 mM) of nitrate and ammonium applied 36 and 39 DAP are shown in Fig. 5. The spectra of nitrate- and ammonium-treated nodules were indistinguishable. The major effect, not readily visible until 48 to 72 hours after treatment, was a reduction in the height of both the near-UV and green-yellow absorbance bands, with no change in

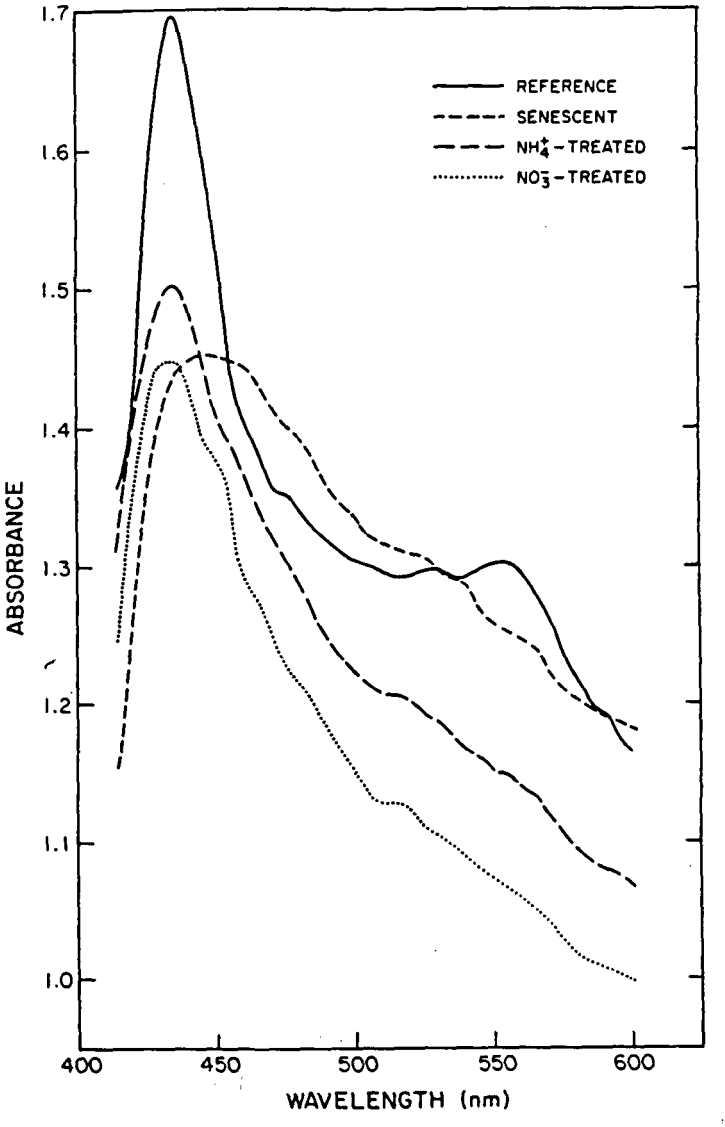

FIGURE 5. Typical Absorbance Spectra of Reference, NO_3^-- and NH_4^+-treated, and Senescent Nodules. Data from Single Replicate Runs Are Chosen as Representative Nodule Spectra. The Spectra of Reference and N-treated Nodules Were Recorded 7 Days After Treatments Were Applied. The Senescent Nodule Spectrum Was Recorded 55 Days After Planting.

the location of the absorbance bands. The data do not support the hypothesis that the mechanism of nitrogen fixation inhibition by nitrate involves the rapid inactivation of Lb by nitrite.

The 510-585 nm absorbance band of untreated nodules disappeared at approximately the same time as flowering (46-48 DAP). After flowering, dissected nodules were found to contain a small spot in the central tissue with the greenish color of legcholeglobin, a breakdown product of leghemoglobin (31). The spectrum of the greenish central tissue of a senescent nodule is shown in Fig. 5. The central tissue of the nitrogen-treated nodules did not develop the color of legcholeglobin during the period of observation, but rather showed a duller pink color than that of the untreated nodules.

The slight color change which resulted from the application of repressive inorganic nitrogen may indicate that these did not cause premature senescence in nodules, although the effects on nodule spectra were similar to those which accompany the breakdown of leghemoglobin into brown pigments. The transformation of leghemoglobin into brown and green hemoglobin pigments during nodule senescence is poorly understood (31, 32), and it is possible that the disappearance of the visible absorbance band as a result of treatment with nitrogen may be related to this transformation. The conversion of leghemoglobin to choleglobin is accompanied by a decrease in the peak height of the near-UV absorbance band and the appearance of a new band at 628 nm (33). Spectra were frequently scanned to 650 nm for evidence of ferrileghemoglobin, which also has an absorbance band in that region, but this absorbance band was not observed in live nodules.

It is possible that the spectral changes caused by treatment with inorganic nitrogen may be related in the short term (1-4 d) to a reduction in nodule respiration rate, and in the long term (4-8 d) to the formation of brown leghemoglobin pigments (34). Treatment with inorganic nitrogen caused a significant reduction both in individual nodule acetylene reduction rates ($p < 0.10$) and in the area of the green-yellow absorbance band (510-585 nm) ($p < 0.05$); however, specific nodule activity did not correlate with either the height or the area of the green-yellow absorbance band.

It is reasonable that the area of the green-yellow absorbance band is an indicator of the rate of nitrogen fixation because it integrates absorption by heme moieties involved in both O_2 transport (i.e. Lb) and electron transport (cyt c), both

of which reflect bacteroid respiration rates (15, 16). Spectra of the cytochrome c-cytochrome oxidase system recorded in both the oxidized and reduced states imply that the height of the 550 nm cytochrome c peak should be greatly diminished when the electron flux through the cytochrome and its oxidase is small (35).

The nitrogenase enzyme is readily inactivated by exposure to oxygen. The puncture of the nodule central tissue by the syringe needles holding the optical fibers could allow oxygen entry, although the vacuum grease applied to the needles probably filled any voids formed as the needles advanced. Only a slight reduction in the height of the green-yellow absorbance band was noted 3-5 h after puncturing the central tissue of nodules still attached to roots. This suggests that there was little degradation of the nodule central tissue during the assays. A disadvantage of the fiber-optic technique is the difficulty of determining the precise location of the nodule central tissue being examined; this problem was avoided by using cowpea nodules of nearly equal physiological age.

CONCLUSION

The repression of nitrogenase activity by nitrate was shown not to be due to the rapid inactivation of leghemoglobin by nitrite, but may result from the much slower inhibition of nitrogenase and leghemoglobin syntheses.

The fiber-optic technique described here can be used to assay any nodule, whether attached or detached from the root. The introduction of artifacts due to the disruption of nodule structure is minimized by the rapidity of the assay (scan times of a few seconds are now possible). Used as a leghemoglobin assay, this technique could significantly reduce the time and greenhouse space required for screening strains of Rhizobium for effectiveness, and for assessing the effect of environmental variables requiring the examination of large numbers of nodules.

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